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Plant Cell Reports

ISSN 0721-7714

Plant Cell Rep
DOI 10.1007/s00299-013-1465-7

Springer

Plant Cell Rep
DOI 10.1007/s00299-013-1465-7

ORIGINAL PAPER

Transcriptome analysis of cytoplasmic male sterility and restoration in CMS-D8 cotton

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Received: 22 April 2013 / Revised: 16 May 2013 / Accepted: 23 May 2013

Abstract
Key message **A global view of differential expression of genes in CMS-D8 of cotton was presented in this study which will facilitate the understanding of cytoplasmic male sterility in cotton.**
Abstract Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants which is incapable of producing functional pollen. However, the male fertility can be restored by one or more nuclear-encoded restorer genes. A genome-wide transcriptome analysis of CMS and restoration in cotton is currently lacking. In this study, Affymetrix GeneChips© Cotton Genome Array containing 24,132 transcripts was used to compare differentially expressed (DE) genes of flower buds at the meiosis stage between CMS and its restorer cotton plants conditioned by the D8 cytoplasm. A total of 458 (1.9 %) of DE genes including 127 up-regulated and 331 down-regulated ones were identified in the CMS-D8 line. Quantitative RT-PCR was used to validate 10 DE genes selected from seven functional categories. The most frequent DE gene group was found to encode putative proteins involved in cell wall expansion, such as pectinesterase, pectate lyase, pectin methylesterase, glyoxal oxidase, polygalacturonase, indole-3-acetic acid-amino synthetase, and xyloglucan endo-transglycosylase. Genes in cytoskeleton category including actin, which plays a key role in cell wall expansion, cell elongation and cell division, were also highly differentially expressed between the fertile and CMS plants. This work represents the first study in utilizing microarray to identify CMS-related genes by comparing overall DE genes between fertile and CMS plants in cotton. The results provide evidence that many CMS-associated genes are mainly involved in cell wall expansion. Further analysis will be required to elucidate the molecular mechanisms of male sterility which will facilitate the development of new hybrid cultivars in cotton.

Keywords Cotton · Cytoplasmic male sterility (CMS) · CMS-D8 · Microarray · Differentially expressed genes

This work is dedicated to the memory of Dr. James McD. Stewart (Department of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, AR, USA) as the developer of the CMS-D8 system in cotton and for his numerous contributions to genetic and genomic studies of the system.

Communicated by V. Srivastava.

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait in >150 higher plants (Zhang and Stewart 2001). CMS plants, while exhibiting normal vegetative growth and female fertility, are incapable of producing functional pollen due to mitochondrial dysfunction during meiosis or microsporogenesis (Dieterich et al. 2003). However, CMS plants are different from other mitochondrial mutants, in that mitochondrial dysfunction in CMS plants is observed in a male-specific manner, while other mitochondrial mutants caused by loss-of-function are not restricted to

Published online: 07 June 2013

male-specific organs, leading to the lethality or developmental growth inability. Therefore, CMS might be gain-of-function instead of a loss-of-function (Fujii et al. 2009). The CMS phenotype is useful for economically producing commercially valuable F_1 hybrid exhibiting hybrid vigor, i.e., heterosis.

In recent years, transcriptomic analysis using DNA microarray and RNA-Seq technology has enabled the analysis and comparison of thousands of genes in one experiment. This has allowed extensive studies to better understand fundamental aspects of growth and development, as well as genetic variation in many plants on a genomic scale (Zenoni et al. 2010). Microarray techniques made from cDNA spotted or unigene EST-based oligo nucleotide systems onto a glass slide have been utilized to conduct comparative genomics as well as gene expression mapping, mutation assay, gene discovery, gene expression profiling, and high-throughput genetic mapping (Zhang et al. 2008). Genes, which are highly responsive to a variety of developmental factors or stress, such as cold stress, wounding, insect feeding, salt, pathogen infection, and drought, have been identified and characterized by this technique (Endo et al. 2002).

A limited microarray studies on CMS have shown the importance of crosstalk between the nucleus and mitochondria in that the nucleus regulates organelle gene expression while signals radiated from organelle, such as mitochondria, also regulate nuclear gene expression, termed retrograde signaling (Fujii et al. 2007). Fujii et al. (2007) conducted a comparative nuclear gene expression study in mature anthers between the CW-CMS line, [cms-CW] *rf17rf17*, and a maintainer line with normal cytoplasm of rice (*Oryza sativa*), [normal] *rf17rf17*. Up-regulation of 58 genes and down-regulation of 82 genes at >threefold changes were observed in the CMS line. Some extremely up-regulated genes in the CMS line included those encoding for alternative oxidase (AOX)1a that participates in cyanide-resistance in the mitochondrial electron chain and removal of reactive oxygen species, protein kinase family, myb family transcription factors, and mRNA binding protein. Down-regulated genes in the CMS line included genes encoding for peroxidase, pectate lyase family protein, chlorophyll A–B binding protein, calcium-binding protein, and MATE efflux family of proteins. Because CMS in plants occurs due to the dysfunction of mitochondrial genes, these results revealed that mitochondrial retrograde pathway may play a role in the change of nuclear-specific gene expression, and this pathway causing an alteration of nuclear gene expression may have special effects on CMS plants. DCW11, down-regulated gene 11 in CW-type CMS rice identified by the above microarray analysis, encoding mitochondrial protein phosphatase 2c, was later shown to be related to CMS (Fujii and Toriyama 2008).

Another microarray-based transcriptomic study conducted by Carlsson et al. (2007) also showed that a substantial number of differentially expressed nuclear genes between CMS line and fertile maintainer line of *Brassica napus* in flower tissue were detected using an *Arabidopsis thaliana* flower-specific cDNA microarray. Nuclear genes involved in protein import into organelles, stamens and pollen-specifically expressed genes, and genes involved in cell wall remodeling were suppressed in the CMS line in comparison with the maintainer line. These results revealed that nuclear gene expression is affected by the mitochondrial genomic dysfunction due to CMS, leading to the suggestion that retrograde signaling crosstalk between nucleus and mitochondria is involved in CMS (Carlsson et al. 2007). More recently, Dong et al. (2013) identified 4,646 differentially expressed genes in floral buds between Ogura-CMS Chinese cabbage and its maintainer line of *B. rapa* ssp. pekinensis using 300-K oligomeric probe (Br300 K) microarrays. Ogura-CMS Chinese cabbage produced few and infertile pollen grains on indehiscent anthers. The Ogura-CMS line had specific gene expression related to stress and redox, but lacked gene expression related to pollen coat and germination. Many nuclear genes related to auxin response, ATP synthesis, pollen development and stress response had delayed expression in Ogura-CMS plants, implying that mitochondrial retrograde signaling suppresses nuclear gene expression.

In Upland cotton (*Gossypium hirsutum*), a non-restoring fertile maintainer ARK8518 (*rf2rf2*) and its isogenic heterozygous D8 restorer line, ARK8518R (*Rf2rf2*) with D8 cytoplasm from *G. trilobum* were utilized to identify differentially expressed genes in anther tissues by mRNA differential display (Zhang et al. 2008). Four genes, including genes encoding a Cys-3-His zinc finger protein and aminopeptidase, were up-regulated, while 22 genes were down-regulated, such as those encoding for phosphoribosylanthranilate transferase (PAT), starch synthase (SS), 4-coumarate-CoA ligase, electron transporter, calnexin, arginine decarboxylase, and polyubiquitin in the heterozygous restorer ARK8518R. The lack of starch accumulation in sterile *rf2* pollen grains in the heterozygous restored plants was caused by the down-regulation of SS. However, there is no comprehensive comparative analysis in CMS-D8 cotton at the genome-wide level using microarray or RNA-Seq.

Male sterility in the CMS-D8 occurs with no pollen production due to the failure of meiosis in the anthers, a typical of sporophytic CMS system. However, its fertility restoration by *Rf2* is a gametophytic system because two types of pollen grains (sterile and fertile in an equal ratio) exist after meiosis in the restored F1 (i.e., CMS line × its restorer line) plants. Zhang et al. (2008) indicated that the CMS-associated gene(s) are expressed in sporophytic issues and the *Rf2* is also not an anther- or pollen-specific

gene. Considering that both the CMS and restorer genes are expressed in both somatic and reproductive tissues and the difficulty in harvesting sufficient amount of anther tissues from young flower buds for RNA extraction, this study compared a global differential gene expression between a CMS line and its isogenic restorer line using flower buds at the meiosis stage. Several CMS candidate genes from differentially expressed patterns between the fertile and sterile line were identified.

Materials and methods

Plant materials

Cotton plants from two isogenic genotypes, CMS-D8 (D8CMS8518) and restorer (D8R8518) on the ARK8518 background were grown in the field of New Mexico State University, Las Cruces, NM. The two lines were isolated by repeated backcrossing to a restorer donor for more than ten generations using ARK8518 as the recurrent parent. The CMS line is sterile with D8 cytoplasm and possesses homozygous recessive fertility restorer alleles (rf_2rf_2). The restorer line is fertile with D8 cytoplasm and possessed homozygous dominant fertility restorer alleles (Rf_2Rf_2) to recover the fertility in cotton plants (Zhang and Stewart 2001).

Total RNA extraction

Due to the small size of flower buds (~3 mm long) and difficulty in harvesting only the male part at the stage of meiosis, flower buds (including petals and styles) were cut above ovaries and immediately frozen in liquid nitrogen when harvesting and stored in a −80 °C freezer. Total RNA was isolated using the "hot borate method" (Wan and Wilkins 1994; Wilkins and Smart 1996) with modifications for micro-preparation, convenience and quickness (Pang et al. 2011). The concentration and purity of total RNA were determined using a DU530 Life Science UV/Vis Spectrophotometer (Beckman Coulter, Brea, CA, USA). The integrity of the total RNA was checked by denaturing formaldehyde agarose (FA) gel electrophoresis and ethidium bromide staining.

Microarrays and data analysis

For the microarray experiments, extracted and purified total RNA from replications of flower buds for sterile D8CMS8518 and fertile D8R8518 lines was pooled in an equal molar ratio. Two milligrams of cleaned total RNA and Affymetrix GeneChips© Cotton Genome Array (Santa Clara, CA, USA) was sent to Genome Explorations (Memphis, TN, USA) for hybridization and preliminary data analysis. A pair-wise comparison between sterile D8CMS8518 and fertile D8R8518 in flower buds of both genotypes was conducted for identification of DE genes. Using the Affymetrix GeneChip Operating Software, the relative mean signal, detection calls, signal log ratios and change calls are independently calculated using four different algorithms for each probe set. Excel files with statistically relevant up-regulated and down-regulated genes and their signal $\log_2$ ratios were generated. The full or partial sequences of DE genes between sterile and fertile plants in flowering buds were retrieved utilizing the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov). The sequences obtained by Blast search in NCBI were utilized for RT-PCR and quantitative RT-PCR to verify DE genes from the microarray analysis. Annotation of unidentified genes by Affymetrix was further manually performed by utilizing the web tools from EasyGO website (http://bioinformatics.cau.edu.cn/easygo/), BLASTx, BLASTn, and UniGene in NCBI database (http://www.ncbi.nlm.nih.gov/unigene/). The genes listed in Tables 2, 3 and 4 were identified and analyzed by those bioinformatics tools. TC (tentative consensus sequence) shown in these Tables were identified using the Cotton Gene Index website (http://compbio.dfci.harvard.edu/tgi/).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to validate the abundance of transcripts observed by the microarray experiment. Nucleotide sequences from a selected subset of highly differentially expressed genes were BLAST searched in the NCBI database to obtain orthologous DNA sequences in *G. hirsutum* genes. The designing of gene-specific primers (Table 1) was accomplished using the IDT PrimerQuest software (Integrated DNA Technologies, Coralville, IA, USA; http://www.idtdna.com/Home/Home.aspx). The primer product sizes ranged from 150 to 200 bp, a suitable size for conducting qRT-PCR analysis.

qRT-PCRs were carried out with the Express one-Step GreenER kit (Invitrogen, Carlsbad, CA, USA) as described in the users' instructions in the iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each real-time PCR assay was performed in technical triplicate for each of the 20 ng/μL, 4 ng/μL, and 800 pg/μL concentrations of total RNA. In addition, no reverse transcriptase and no template controls were added on a Bio-Rad iQ5 Thermal Cycler (Hercules, CA, USA) to check any DNA contamination. One-step RT-PCRs of 20 μL volume were conducted, consisting of 10 μL EXPRESS SYBR GreenER qPCR SuperMix Universal (Invitrogen, Carlsbad, CA, USA), 20 nM Fluorescein reference dye (Invitrogen, Carlsbad, CA, USA), 0.5 μL EXPRESS SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 0.2 μM forward and

Table 1 The forward and reverse primer sequences used for qRT-PCR

Probe set ID	EST ID	Tentative function	Fold change	Functional categories	Expected size (bp)	Primer sequences
Ghi.4013.1.A1_at	CO492517	CITT	−5.5	Transporter	128	F:CCGACGATGCATTTAGCAACCCTT
						R:TACCATGGCGTTCTTCTTCTCCGT
Ghi.8355.1.A1_at	CA992719	Heat shock protein	−5.6	Environmental stress	194	F:TATCCACATTCGCAGGCAACCTGA
						R:GTACCATGCCATTACCCTGGACAT
Ghi.8043.1.S1_x_at	U09717.1	Polygalacturonase	−5.9	Cell wall metabolism	193	F:GTTTATTTGCAGCGGTTCTTCGCC
						R:TGTTGGTATGCTTTAAGCGGTGGC
Gra.2138.2.A1_at	CO127790	Pectin methylesterase	−6.1	Cell wall metabolism	186	F:ACTCTTGCATCGAGGTTAGCACCA
						R:TAAGTTCCCGACATACTTGGGCAG
Ghi.468.1.A1_s_at	AY189970.1	Profilin	−6.1	Cytoskeleton	189	F:TCATCACCTTTCTGCCGCTGCTAT
						R:ACCATAAACTTTGCGCCACCAAGG
Gra.2350.1.A1_at	CO124161	Glyoxal oxidase	−6.5	Cell wall metabolism	199	F:ATCGACGGCAAGGTACTCCATTGT
						R:ACGATTTACTCGCCGCCATTTGTG
Gra.1810.2.S1_at	CO125520	Pectinesterase	−8.1	Cell wall metabolism	175	F:CCGTGTGCTGTAACCATGTTGTGT
						R:TCACTGGGTACCAAGACACCCTTT
Gra.2056.1.A1_s_at	CO121156	Beta-galactosidase	−8.3	Carbohydrate metabolism	200	F:TTAGGCACATTTGACTTGGACGGC
						R:GCTATGGTGATCCATTTGGCGCTT
Ghi.824.1.S1_at	DR454424	Pollen coat-like	−7.2	Other	175	F:AACCAATCCCAGGATTTGAGCCAC
						R:CCTTTATGGCATCTGTTGCGCCTT
GhiAffx.43252.1.S1_at	DW512213.1	Cytochrome c	−4.2	Energy metabolism	127	F:GCACGTTCCTGTGGCTTCTTCAAT
						R:CTGCAAACAAGAGCATGGCTGTCA

CITT cadmium ion transmembrane transporter

reverse primers, 1.5 μL RNA template and 3.2 μL sterile water (Promega, Madison, WI, USA). Reactions were run using the pre-set one-step RT-PCR with melt curve program. Reaction conditions were as follows: 50 °C for 10 min for cDNA synthesis, 95 °C for 5 min for denaturing Reverse Transcriptase, followed by amplification reaction; 45 cycles of 95 °C for 10 s. and 60 °C for 30 s., and ending with the melt curve program. The Bio-Rad iQ5 optical system software was utilized for calculation of the relative quantification and standard deviation for statistical analysis as described in the iQ5 system software instruction manual (Bio-Rad, Hercules, CA, USA). qRT-PCR data were analyzed with the iQ5 Optical System Software (Bio-Rad, Hercules, CA, USA). A melting curve was calculated for each qRT-PCR to determine PCR performance, and the amplification of a single product was verified by gel electrophoresis. The data normalization of expression of each gene was conducted in relation to its control (fertile plants).

Results

Differentially expressed genes in CMS-D8 flower buds based on microarray analysis

In this study, due to the difficulty in harvesting only anthers at the meiosis stage, flower buds without ovaries were used instead. The strategy was justified because both the CMS and restorer genes are expressed in both sporophytic and gametophytic tissues (Zhang et al. 2008). We compared DE genes of flower buds at the meiosis stage between CMS and its restorer cotton plants conditioned by the D8 cytoplasm utilizing the Affymetrix GeneChips© Cotton Genome Array. The signal intensities of the 24,132 transcripts in flowering buds were compared between the CMS line and its isogenic fertile line, resulting in a total of 458 DE genes (1.9 % of all the genes) identified. Of the 458 DE genes, 127 were up-regulated and 331 were down-regulated; and 102 of genes were up-regulated and 103 genes were down-regulated at >twofold changes. The DE genes between the CMS (sterile) and fertile plants were further categorized based on their functions. The majority of unclassified categories provide a valuable opportunity for functional analysis to better understand specific gene regulatory systems in the flower buds and to discover novel genes involved in formation of fertile microspores or pollen grains. The putative annotated protein products of the DE genes in flowering buds between sterile and fertile plants were composed of the following categories. Among the down-regulated genes, 72 (22 %) DE gene products had unknown or unclassified functions (Fig. 1a) and 61 (19 %) products belonged to the other categories. These other categories consisted of 43 genes for signaling (13 %), 41 genes for other metabolism (12 %), 27 genes for cell

wall metabolism (12 %), 25 genes for carbohydrate metabolism (8 %), 24 genes for transport (7 %), 21 genes for energy metabolism (6 %), and 17 genes for transcription (5 %). For up-regulated genes (Fig. 1b), there were 47 genes for unclassified products (37 %), 22 genes for other metabolisms (17 %), 20 genes for signaling (16 %), 11 genes for other categories (9 %), 10 genes for transcription (8 %), 7 genes for cell wall metabolisms (5 %), 6 genes for transport (5 %), and 4 genes for carbohydrate metabolisms (3 %).

Quantitative RT-PCR of genes from seven different functional categories

As shown in Fig. 2, qRT-PCR analysis of 10 genes that were selected according to highly DE microarray profiles and putative functions confirmed the corresponding microarray results. A cadmium ion transmembrane transporter represents transporter category and a heat shock protein for environmental stress. Four gene products, i.e., polygalacturonase, pectin methylesterase, glyoxal oxidase, and pectinesterase family protein, are responsible for cell wall metabolism; profilin for cytoskeleton; beta-galactosidase for carbohydrate metabolism; pollen coat-like protein for other categories; and cytochrome c for energy metabolism. The results clearly showed similar expression patterns for all of the tested genes between microarray and qRT-PCR analyses. The $\log_2$ ratios from the microarray analysis and the qRT-PCR fold changes, Probe Set ID, EST ID, tentative function, functional categories of selected genes, expected amplified sizes for qRT-PCR, and reverse and forward primer sequences for these genes are shown in Table 1. All the selected genes showed stronger gene expression in flowering buds of fertile plants compared to those of the CMS line. The qRT-PCR results demonstrated that the microarray results were reliable for further analysis.

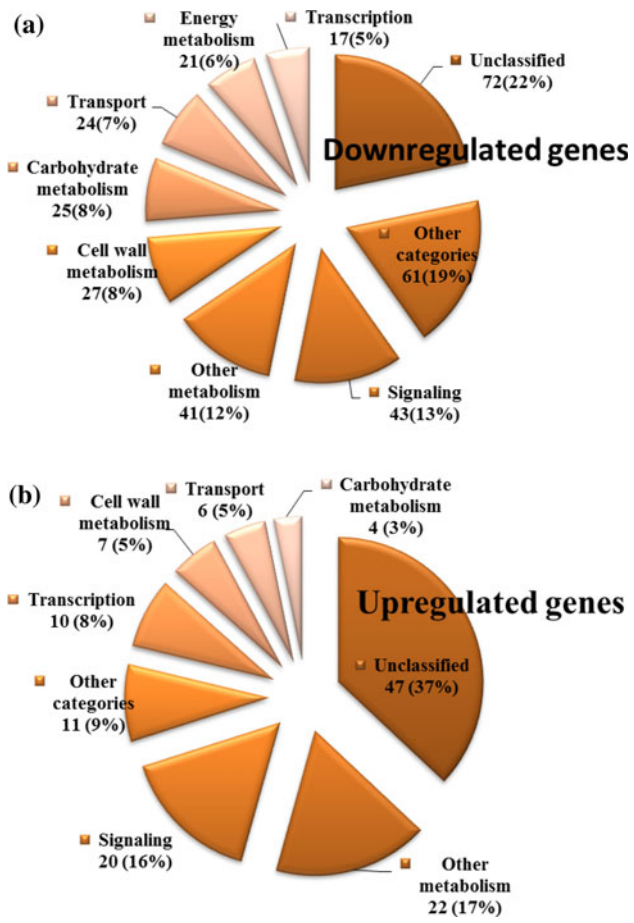

Fig. 1 Functional categories of differentially expressed genes in comparison between CMS line and fertile line in flower buds. **a** Down-regulated genes. **b** Up-regulated genes

Putative functions of differentially expressed genes between sterile and fertile flowering buds

A group of highly DE genes ($n = 27$, 8 %; Table 2) encoded for the putative proteins that are involved in cell wall expansion, such as pectinesterase (CO125520; CO124798; CO117773; CO121526; CO117349; DW51 3914; and DR460203), which plays a role in plant cell wall modification and subsequent breakdown (Carpita and Gibeaut 1993). Other genes such as those encoding for pectate lyase (CO117467: DT048897), pectin methylesterase (CO127790), glyoxal oxidase (CO124161), polygalacturonase (U09717.1), indole-3-acetic acid-amino synthetase (DW227879), xyloglucan endo-transglycosylase (DW22 7168), and protease inhibitor/seed storage/lipid transfer protein (LTP) (CD485893: CD486563: DT554276) are related to cell wall metabolism. This is mainly due to the fact that the cell wall was loosened for cell wall expansion, resulting in the formation of functional pollen (Campbell and Braam 1999). However, these cell wall-related genes were down-regulated in the CMS line which does not produce functional pollen. The genes encoding for pectinesterase were one of the examples in down-regulated genes in the CMS line, which is responsible for controlling relevant physiological processes, such as the cell expansion during cell growth and fruit ripening (Willats et al. 2001; Carpita and Gibeaut 1993). Polygalacturonase was also one of the highest down-regulated genes in the sterile line, which plays a role in the breakdown of pectin and is consequently involved in the stages of plant development (Hadfield and Bennett 1998) as well as pollen grain maturation and pollen tube growth (Jiang et al. 2008). Xyloglucan endo-transglycosylase (XET), which plays a significant role by allowing the cell wall to be loosened for cell wall expansion (Campbell and

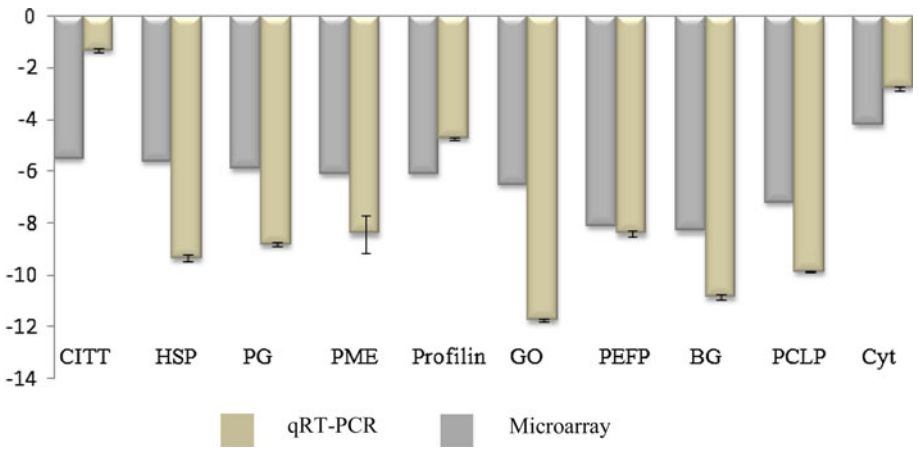

Fig. 2 Quantitative RT-PCR analysis on ten significantly down-regulated genes based on the microarray data set. The transcript abundance of sterile plants compared with the $\log_2$ ratio is utilized to show fold changes of both microarray and qRT-PCR. *CITT* cadmium ion transmembrane transporter, *HSP* heat shock protein, *PG* polygalacturonase, *PME* pectin methylesterase1, Profilin, *GO* glyoxal oxidase-related, *PEFP* pectinesterase family protein, *BG* beta-galactosidase, *PCLP* pollen coat-like protein, *Cyt* cytochrome c

Braam 1999; Pauly et al. 2001; Bourquin et al. 2002), was down-regulated in the CMS line. These results indicated that pollen-specific regulatory genes and cell expansion-related genes are transcriptionally highly active in flower buds of fertile plants. Genes in a variety of functional categories, including cell wall metabolism, carbohydrate metabolism, cytoskeleton, energy metabolism, lipid metabolism, signaling, transcription, and transporter, were also down-regulated in the CMS genotype.

Genes in the cytoskeleton category also play a key role in assisting cell wall expansion (Table 3). Actin 12 (DT567198), actin 11 (AI726503; CO124794; DV849072) and actin (DR459315: DT048095: DN759693: CO12 5023: CO125023) were down-regulated. These genes play a role in the cytoskeleton as a microfilament and are involved in many important functions including cell elongation, cell division, and cell mobility (Blanchoin et al. 2010). Profilin (AY189970), which is responsible for cell elongation, cell shape maintenance, determination of flowering time and polarized growth of root hair and trichomes (Ramachandran et al. 2000), was highly down-regulated in the CMS line. Alpha-tubulin (AY345605) and beta-tubulin (AI726113; DT557030), which are the basic component of microtubules, were also down-regulated in the CMS line.

In energy metabolisms, 20 genes were down-regulated in the CMS line in comparison to the fertile line (Table 4). One of the mitochondrial genes cytochrome *c* (DW512213; CF075617; DW500449) involved in electron transport chain, and adenylate kinase (DW237831) implicated in reporting of signals to metabolic sensors in the inner membrane of the mitochondria (Dzeja and Terzic 2009), was highly down-regulated in flowering buds of the CMS line. A 14-3-3b protein (DV849560), which plays a role in signaling, cell growth, division, stress response and ion-channel regulation (Aitken 2006), was down-regulated in the CMS plant. Bunney et al. (2001) demonstrated that 14-3-3 proteins are involved in the regulation of ATP synthases in plant mitochondria within the inner membrane compartment in a phosphorylation-dependent manner. The gene coding for sulfite oxidase (CO499223), which is localized in the mitochondria of all eukaryotes, was also one of the highest down-regulated genes. This enzyme oxidizes sulfite to sulfate and via cytochrome c, transfers the electrons produced to the electron transport chain, allowing the generation of ATP in oxidative phosphorylation (Lee et al. 2002; Feng et al. 2003). A blue copper protein (CO494805) was down-regulated in the CMS line. The protein, which is also known as cupredoxins, is found in the electron transport chain of prokaryotes and eukaryotes to play a role in shuttling electrons from an electron donor to an electron acceptor in bacteria and plants (De Rienzo et al. 2000; Klinman 1996).

In addition, some of the stress-related genes expressed in the fertile plants encode proteins known to protect plants from environmental stress. For example, a gene-encoding heat shock proteins (CA992719, TC263484) in the CMS line was highly down-regulated (by 5.6-fold). The proteins are recognized as a family of proteins that function as molecular chaperones by preventing protein aggregation across the cell membrane and are also known to be involved in the fortification of pollen tube which is particularly sensitive to temperature changes (Ahmed et al. 1992; Sangster et al. 2007; Baniwal et al. 2004; Queitsch et al. 2000, 2002). Another highly down-regulated gene (by 7.2-fold) in the CMS line, encoding pollen coat-like

Table 2 Functional classification of cell wall metabolism based on the down-regulated genes in CMS line in comparison to fertile line in cotton flower buds

Probe set ID	GeneBank	TC	Annotation	Log_2 ratio
Ghi.9475.1.S1_s_at	DR460697	TC156663	Pectinesterase inhibitor	−1
Ghi.7226.1.S1_s_at	AW186996	TC171792	Arabinogalactan protein	−1.1
GhiAffx.6110.1.A1_x_at	DW226506	TC158611	Fascilin-like arabinogalactan protein	−1.2
Ghi.10002.1.S1_at	DN758503	TC134191	Polygalacturonase	−1.2
GhiAffx.5900.1.S1_at	DR460203	TC160274	Pectinesterase	−1.3
Gra.123.2.S1_s_at	CO087068	TC140969	Cinnamyl-alcohol dehydrogenase	−1.3
Ghi.249.1.A1_at	DQ204496	N/A	Alpha-expansin	−1.4
Ghi.4318.2.A1_s_at	DT054325	Singleton	GH3.1 (indole-3-acetic acid-amido synthetase)	−1.5
Ghi.10143.1.S1_at	DT554276	N/A	Protease inhibitor/seed storage/lipid transfer protein (LTP)	−1.7
GraAffx.28690.1.S1_s_at	CO085760	TC152132	Pectinacetylesterase	−1.7
Ghi.4866.1.A1_s_at	DT048897	TC135792	Pectate lyase	−1.9
GhiAffx.6395.1.S1_s_at	DW484802	TC148549	IAA14 (indole-3-acetic acid inducible 14)	−2.4
Ghi.10795.1.S1_s_at	CD486563	TC153415	Protease inhibitor/seed storage/lipid transfer protein (LTP)	−2.4
Ghi.6472.1.A1_s_at	CD485893	TC162624	Protease inhibitor/seed storage/lipid transfer protein (LTP)	−2.8
GhiAffx.43551.1.S1_x_at	DW513914	TC149514	Pectinesterase	−3.2
Ghi.8147.1.S1_at	DQ073046	N/A	Pectate lyase	−3.3
GhiAffx.15786.1.S1_s_at	DW227879	Singleton	GH3.1 (indole-3-acetic acid-amido synthetase)	−3.4
Gra.1786.1.S1_at	CO117349	TC138346	Pectinesterase	−3.5
Gra.2151.1.A1_at	CO121526	TC146000	Pectinesterase	−4.4
Gra.1669.1.A1_at	CO117773	TC140178	Pectinesterase	−4.9
Gra.1177.1.A1_at	CO124798	TC134527	Pectinesterase	−4.9
GhiAffx.29764.1.S1_at	DW227168	TC160452	Xyloglucan endo-transglycosylase	−5
Ghi.8043.1.S1_x_at	U09717.1	N/A	Polygalacturonase	−5.9
Gra.2138.2.A1_at	CO127790	Singleton	Pectin methylesterase	−6.1
Gra.2350.1.A1_at	CO124161	TC138276	Glyoxal oxidase-related	−6.5
GraAffx.22776.1.A1_at	CO117467	TC146209	Pectate lyase	−6.9
Gra.1810.2.S1_at	CO125520	TC146620	Pectinesterase	−8.1
Gra.623.2.S1_s_at	CO090980	TC134856	Xyloglucan endotransglucosylase	1.5
Gra.1600.1.A1_at	CO120161	TC131422	Cellulose synthase catalytic subunit	2
Ghi.8914.1.A1_at	DT457861	TC139406	Proline-rich cell wall protein	2.1
GraAffx.17334.1.A1_at	CO101426	TC143599	Alpha-expansin	2.7
Ghi.5695.1.A1_at	DT046736	TC169017	Indole-3-acetic acid-amido synthetase GH3.17	2.8
Gra.1367.1.A1_s_at	CO111879	TC148150	Hydroxyproline-rich glycoprotein family protein	3.7
Gra.448.1.A1_at	CO098818	TC130508	Xyloglucan endotransglucosylase	5.4

proteins (DR454424, TC264767), is reproduction/development-related and dysfunctions of this protein cause elimination of pollination completely (Mayfield et al. 2001). A homologous Bcp1 protein gene required in pollen fertility in Arabidopsis (Xu et al. 1995) was also down-regulated (by 2.9-fold) in the CMS flowering buds. These results suggest that acquisition of the functional pollen in fertile flowering buds depends on the tight temporal regulation of a number of genes in a tissue-specific manner, and requires the up-regulation of specific genes involved in cell wall enlargement, cell shape organization, modification, energy supply and pollen-specific genes.

Discussion

The development of functional pollen and their release timing at the specific stage to maximize pollination is important for plant reproduction and genetic diversity. The timing of anther development and microsporogenesis stages is tightly regulated and is characterized by specific phenomenon from initial cell differentiation of the floral meristem to pollen formation, maturation, and release during anther dehiscence events (Goldberg et al. 1993; Sanders et al. 1999; Scott et al. 2004; Ma 2005). The deposition of the pollen wall is of great importance during

Table 3 Functional classification of cytoskeleton based on the down-regulated genes in CMS line in comparison to fertile line in cotton flower buds

Probe set ID	GeneBank	TC	Annotation	Log_2 ratio
Ghi.9969.3.A1_s_at	AI726205	TC141614	Tubulin, beta 2	−1
AFFX-Gra-actin-M_s_at	CO125023	TC140485	Actin	−1
GraAffx.16191.1.S1_x_at	CO125023	TC140485	Actin	−1
Ghi.9969.2.S1_s_at	DT557030	N/A	Beta-tubulin	−1.1
Ghi.9969.1.A1_x_at	AI726113	TC171815	Beta-tubulin	−1.2
Ghi.1314.1.S1_x_at	AY345605	N/A	Alpha-tubulin	−1.2
AFFX-Gra-actin-3_x_at	CO125023	TC140485	Actin	−1.3
Ghi.1723.1.S1_s_at	DN759693	TC140485	Actin	−1.4
Ghi.10494.1.S1_s_at	DT048095	TC143655	Actin	−1.5
Ghi.4663.1.A1_x_at	DT051323	Singleton	Alpha-tubulin	−1.6
Ghi.1314.2.S1_s_at	AY345603	N/A	Alpha-tubulin	−1.7
Ghi.5940.1.A1_at	DR459315	TC165808	Actin	−2
Ghi.1927.1.S1_at	DV849072	TC172092	ACTIN-11	−2.3
Gra.1375.1.A1_at	CO124794	TC166470	ACT11	−3.6
Ghi.7783.1.A1_s_at	AI726503	TC155341	ACT11	−4
Ghi.9363.1.S1_at	DT567198	N/A	ACT12	−5.2
Ghi.468.1.A1_s_at	AY189970	N/A	Profilin	−6.1
Ghi.7687.1.S1_at	DT467528	TC145442	Actin depolymerizing factor	1.6
GraAffx.30915.1.A1_at	CO079320	TC144771	ATMAP65-6 microtubule binding	3.4

Table 4 Functional classification of energy metabolism based on the down-regulated genes in CMS line in comparison to fertile line in cotton flower buds

Probe set ID	GeneBank	TC#	Annotation	Log_2 ratio
GhiAffx.16162.1.A1_s_at	DW227850	TC138233	NDB2 (NAD(P)H dehydrogenase B2); disulfide oxidoreductase	−1
Ghi.4503.1.A1_at	DR457189	TC170136	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	−1
Ghi.8303.1.S1_s_at	DV849178	TC140372	ATP synthase subunit d	−1
Ghi.488.1.S1_s_at	DR459014	TC142000	NADH-ubiquinone oxidoreductase 24 kDa subunit	−1
GhiAffx.6110.2.S1_x_at	DW496609	TC154169	Photosystem I reaction center subunit II, chloroplast precursor	−1
GhiAffx.61573.1.S1_at	DW498498	TC160898	Ubiquinol-cytochrome C reductase	−1.1
GhiAffx.22444.1.S1_at	DW500755	Singleton	Plastocyanin-like domain-containing protein	−1.1
Ghi.5538.1.S1_s_at	DT051061	TC136016	NADH dehydrogenase	−1.1
GhiAffx.60604.1.S1_s_at	DW500449	TC158619	Cytochrome c	−1.2
Ghi.5431.1.S1_s_at	CO496782	TC161225	Cytochrome c oxidase subunit Vc	−1.2
Ghi.6468.1.S1_s_at	CD486706	TC138446	Isocitrate dehydrogenase	−1.2
Ghi.4822.1.A1_s_at	CF075617	TC134389	Cytochrome c	−1.4
GhiAffx.42690.1.S1_s_at	DW514891	TC172066	Mitochondrial glycoprotein family protein/MAM33	−1.7
Ghi.4332.1.A1_s_at	DT052758	TC171354	Fumarase 1	−1.7
Ghi.6104.1.A1_at	CO494805	TC174121	Blue copper-like protein	−2
Ghi.5852.2.A1_s_at	CO499223	TC178457	SOX (sulfite oxidase)	−2.6
Ghi.3524.2.A1_at	DT468351	TC173686	NDA2 (alternative NAD(P)H dehydrogenase 2)	−2.8
Ghi.7445.1.S1_x_at	DV849560	Singleton	14-3-3b protein	−2.9
GhiAffx.3488.1.S1_at	DW237831	Singleton	Adenylate kinase	−3.4
GhiAffx.43252.1.S1_at	DW512213	TC156698	Cytochrome c	−4.2

pollen maturation for pollen protection, dispersal, and pollen-stigma recognition (Piffanelli et al. 1998; Scott et al. 2004). It was reported that mutated *Arabidopsis* plants in three cellulose synthase CESA genes involved in primary wall formation are male sterile and show aberrant pollen wall formation. The pollen wall is composed of multiple

layers and is among the most complex plant extracellular matrices (Blackmore et al. 2007). Cellulose deposition by the haploid microspores is of significant importance for pollen wall development and pollen fertility (Persson et al. 2007; Vizcay-Barrena and Wilson 2006; Zhang et al. 2006). Numerous male sterile or partially sterile mutants that have been isolated and characterized in *Arabidopsis* define some of the key processes in anther and male gametophyte development (Taylor et al. 1998; Sanders et al. 1999; Ma 2005).

Genetic analyses and transcriptomics studies in *A. thaliana* and *Zea mays* open the door for dissecting pollen development and function. Pollen transcriptome analysis was reported for various stages of pollen development (Becker et al. 2003; Honys and Twell 2003, 2004; Pina et al. 2005; Schmid et al. 2005). Transcriptomic experiments in *Arabidopsis* using staged pollen identified 13,977 genes that were expressed in the male gametophyte during development (Honys and Twell 2004). This clearly indicated the diversity and extent of gene expression during anther and pollen development. It was reported that many phytohormones and a variety of biosynthetic or signaling mutants of phytohormones including auxin, gibberellins, ethylene, cytokinins, and jasmonic acids can affect male fertility (Huang et al. 2003; Cecchetti et al. 2008; Kieber et al. 1993; Park et al. 2002; Singh et al. 2002). The involvement of plant hormone auxin in every aspect of plant growth and development has been reported (Dharmasiri et al. 2005). The responsibility of auxin is to foster the degradation of transcriptional regulators called Aux/IAA proteins. Aux/IAA degradation requires TIR1, one of the F box proteins, which serves as an auxin receptor. Three additional F box proteins, called AFB1, 2, and 3, also regulate auxin response. It was demonstrated that auxin biosynthetic double mutants *yuc2 yuc6* (Cheng et al. 2006) and auxin signaling quadruple mutant *tir1afb1-1*, *afb2-1*, *afb3-1* plants (Dharmasiri et al. 2005) dramatically decreased male fertility.

Mutations in hormone pathways of gibberellins (Cheng et al. 2004), ethylene (Kieber et al. 1993), and jasmonic acid (Park et al. 2002) also result in the reduction of male fertility.

DELLA proteins, RGA, RGL1, and RGL2 inhibit stamen growth and anther development in the ga1-3 mutant, which exhibits male sterility (Cheng et al. 2004).

Controlling the process of pollen development and release is required for selective breeding, the release of genetically modified (GM) pollen, and the commercial development of hybrid lines by utilizing heterosis or hybrid vigor. CMS system has been utilized in many crops for the development of higher yield, commercially superior crops (Hanson and Bentolila 2004; Pelletier and Budar 2007). CMS phenotypes occur due to the aberrant open reading frames (orfs) in mitochondrial genomes (Hanson and Bentolila 2004; Pelletier and Budar 2007). The presence of nucleus-encoded fertility restorer gene(s) can restore the plant fertility through interaction between nucleus and mitochondria (Wang et al. 2006). Thus, fertility control can be accomplished by selecting the appropriate breeding lines. However, Texas male sterile (T) cytoplasm in maize includes the unusual mitochondrial chimeric gene URF13 which induces the increased susceptibility to the pathogen, Southern corn leaf blight (*Bipolaris maydis*) race T (Huang et al. 1990; Wise et al. 1999). The understanding of pollen development mechanism and identification of CMS causal genes are required to utilize fertility control to increase the yield and widespread application of this excellent phenomenon.

Studies conducted on *Arabidopsis* plants reveal that coordinated expression of the *B. campestris* pollen protein1 (BCP1) can be specifically down-regulated by the antisense RNA approach (Murray and Crockett 1992) and transgenic study in *Arabidopsis* indicated that such aggravation of BCP1 shows a halt in pollen development through pollen abortion or rejection (Xu et al. 1995). The BCP1 gene, therefore, shows a strong correlation with the production and delivery of male gametes to the sperm sac, as is the known process in plants (Knox 1984). Reports on BCP1 reveal specific gene expression in the diploid tapetum and haploid microspores of the anther wall (Theerakulpisut et al. 1991), where proteins and other molecules are developed and processed to complete proper pollen creation (Scott et al. 1991; Pacini 1990). In our microarray study, BCP1-related gene transcripts retrieved from EST sequences (AI729387) were highly down-regulated in CMS line, showing their involvement in pollen development and structural formation in the cotton plant.

This study employed microarray analysis to reveal differential expression pattern of several categorized gene clusters in cotton anthers of fertile and CMS plants. Based on the expression patterns, we identified numerous anther-specific DE genes in Upland cotton between fertile and CMS plants. This result indicated that the presence of the nucleus-encoded fertility restorer genes not only restores functional pollen formation, but also affects the gene expression of numerous nucleus-encoded genes, including such functional categories as cell wall metabolism, cytoskeleton, energy metabolism, signaling, nucleic acid metabolism, translation or translation factor, lipid metabolism, amino acid metabolism, carbohydrate metabolism, transcription factors, transporter/transport, protein metabolism and environmental stress. In addition, among a wide range of DE genes, some genes were characterized as unknown/uncategorized functional genes, which may lead to discovery of novel CMS-associated genes by the subsequent study. To accomplish the identification of DE

unknown genes, the utilization of T-DNA and transposon tagging may be advantageous to uncover the function and mechanism of these specific genes (Amagai et al. 2003; Tissier et al. 1999). If the function of a specific CMS inducible gene in cotton could be identified from the CMS line, it may be convenient and valuable to produce analogous CMS plants in other related plants since orthologous or homologous CMS-associated genes in other plants may also be exclusively expressed (Amagai et al. 2003). Moreover, a reverse genetics approach employing RNAi or antisense techniques will be beneficial in the characterization of these novel genes. The further characterization of the expression patterns of these genes using in situ hybridization as well as proteomics analysis may also facilitate understanding of their functions in plant male sterility. Till now, transcriptomic analysis for an attempt to identify CMS-associated genes was only conducted in rice and *Brassica* (Fujii et al. 2007; Carlsson et al. 2007; Dong et al. 2013). In summary, the present study is the first report in comprehensive transcriptomic profiling of CMS-associated genes in cotton. We identified a substantial number of DE genes between CMS and fertile plants. Further studies will be required to maximally utilize the valuable tool, CMS system, to obtain more valuable and useful crops possessing the desirable superior traits for plant breeding program.

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